

Choroidal thickness after intravitreal ranibizumab injections for choroidal neovascularization

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Purpose: To study changes in choroidal thickness with ranibizumab treatment for choroidal neovascularization (CNV).

Design: Prospective case series.

Methods: This prospective study consisted of 60 CNV-affected eyes of 60 patients treated with intravitreal injections of ranibizumab using an on-demand protocol after an initial loading phase. The eyes studied included 20 with age-related macular degeneration (AMD), 20 with polypoidal choroidal vasculopathy (PCV), and 20 with myopic CNV. In the eyes with AMD and PCV, choroidal thickness at the fovea was measured with optical coherence tomography using enhanced depth imaging. In eyes with myopic CNV, the choroidal thickness was measured using standard optical coherence tomography without the enhanced depth imaging technique.

Results: With ranibizumab treatment, central retinal thickness decreased significantly ($P < 0.001$) and visual acuity improved significantly ($P < 0.001$). However, central choroidal thickness ($167.2 \pm 108.3 \mu\text{m}$) showed no significant change at 1 month after the loading phase ($165.2 \pm 107.8 \mu\text{m}$, $P = 0.120$) or at final examination ($164.8 \pm 107.7 \mu\text{m}$, $P = 0.115$). At baseline, central retinal thickness in eyes with AMD was significantly greater than those with PCV ($P = 0.005$) or high myopia ($P = 0.029$). However, central choroidal thickness in eyes with myopic CNV was significantly thinner than in eyes with AMD ($P < 0.001$) or PCV ($P < 0.001$). In each type of disease, there was no significant change in central choroidal thickness with ranibizumab treatment.

Conclusion: The effect of ranibizumab on the choroidal thickness is minimal, if any.

Keywords: choroidal thickness, ranibizumab, optical coherence tomography

Introduction

Ranibizumab, a Fab fragment of a recombinant, humanized, monoclonal antibody, blocks all isoforms of vascular endothelial growth factor (VEGF)-A.¹ The inhibition of VEGF-A reduces the leakage from choroidal neovascularization (CNV) and further growth of CNV.^{2,3} The current standard treatment for exudative age-related macular degeneration (AMD) is intravitreal injections of ranibizumab.⁴ Previous large-scale, prospective, randomized studies of eyes with AMD showed dramatic effects of ranibizumab on CNV and subsequent improvement of visual acuity.^{5,6} However, most patients with AMD require frequent injections of ranibizumab to maintain the initial visual improvement during the loading phase.⁷ Mendrinós et al⁸ recently reported that the retinal arteriolar diameter was reduced by 18.5% at 30 days and by 19.1% at 12 months after the initiation of ranibizumab treatments for exudative AMD. Because VEGF has various physiological effects on eyes, including cell proliferation,⁹ neuroprotective

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effects,¹⁰ maintenance of the choriocapillaris,¹¹ and increases in ocular blood flow,¹² long-term inhibition of VEGF may adversely affect the eye.¹³

The choroid is a multifunctional structure with one of the highest blood flow rates in the body.^{14,15} It primarily works to support the function and nutrition of the outer retina, including photoreceptors and retinal pigment epithelium.¹⁵ Since Spaide et al¹⁶ introduced enhanced depth-imaging optical coherence tomography (EDI-OCT), which is based on spectral-domain OCT technology, many investigators have reported on the role of the choroid in the pathogenesis of various ocular diseases,^{17–22} including AMD,^{23–25} polypoidal choroidal vasculopathy (PCV),^{23,24,26} central serous chorioretinopathy,^{27,28} and myopic CNV.^{29,30} It has recently been suggested that choroidal thinning may be involved in the development of CNV associated with AMD and high myopia.^{23,24,31} VEGF works to induce vessel dilation and to increase ocular blood flow through a mechanism involving increased nitric oxide production.^{32,33} It is then possible that long-term inhibition of VEGF by ranibizumab might cause the constriction of the choroidal vessels, leading to further thinning of the choroid. Thus far, however, only limited information is available on the effects of ranibizumab on the choroid. In this prospective study, we performed longitudinal measurements of choroidal thickness before and after ranibizumab treatment for CNV associated with AMD, PCV, and high myopia to evaluate the possible adverse effects of ranibizumab on the choroid.

Patients and methods

This prospective study consisted of 60 eyes of 60 patients with CNV treated with intravitreal injections of ranibizumab (Lucentis; Novartis, Bülach, Switzerland). The choroidal thickness of the eyes was examined using OCT at Kyoto University Hospital between May 2010 and August 2011. The underlying pathology of the 60 eyes included 20 consecutive eyes with AMD, 20 with PCV, and 20 with myopic CNV. The inclusion criteria for the study groups were (1) symptomatic neovascular lesions beneath the center of the fovea, (2) the presence of active exudative features involving the macula, (3) treatment by intravitreal injections of ranibizumab after the initial diagnosis, and (4) OCT examination before and after treatments. Exclusion criteria were (1) the obscuration of retinal or choroidal images by media opacity or thick subfoveal hemorrhage, (2) a history of intraocular inflammation, (3) the presence or history of other macular abnormalities (eg, idiopathic CNV, retinal angiomatous proliferation, angioid streaks, other secondary

CNV, or central serous chorioretinopathy), (4) a history of previous treatment for CNV, and (5) eyes with a history of pars plana vitrectomy or other intraocular surgeries, except history of cataract surgery. Subjects with systemic diseases or conditions that could affect retinal or choroidal thickness were also excluded, such as those with Vogt-Koyanagi-Harada disease, malignant hypertension, or pregnant women.

At the initial visit, each patient underwent a comprehensive ophthalmologic examination, including measurement of best-corrected visual acuity with a Landolt chart; determination of intraocular pressure; indirect ophthalmoscopy; slit lamp biomicroscopy with a contact lens; OCT examination (Spectralis HRA+OCT; Heidelberg Engineering, Heidelberg, Germany); and fluorescein and indocyanine green angiography (HRA-2; Heidelberg Engineering). The diagnostic criteria for PCV were based on indocyanine green angiography, which showed a branching vascular network that terminates in polypoidal swelling. The diagnosis of myopic CNV was based on a minimum spherical equivalent refractive error of more than -6.0 diopters or an axial length of more than 26.5 mm, characteristic retinal signs of high myopia, and evidence of CNV based on the presence of leakage on fluorescein angiography and/or intraretinal or subretinal fluid on OCT.

At the loading phase, all eyes with PCV and AMD received three successive intravitreal injections of ranibizumab at monthly intervals (at baseline and at 1 and 2 months); eyes with myopic CNV were treated by a single injection of ranibizumab. After the loading phase, each patient was scheduled for an examination each month, at which time they underwent a comprehensive ophthalmologic examination. Each eye was re-treated by an intravitreal injection of ranibizumab as an on-demand protocol, depending on the presence of exudative signs, such as intraretinal edema or subretinal fluid on OCT and/or leakage on fluorescein angiography during follow-up. Injections of ranibizumab were performed under sterile conditions, and prophylactic topical antibiotics were applied for 1 week after the injection.

In eyes with AMD and PCV, the choroidal thickness was measured using the EDI technique, which was performed by placing the OCT instrument close enough to the eye to obtain an inverted image. All images were obtained using an eye-tracking system, and 100 scans were averaged automatically to improve the signal-to-noise ratio. The central choroidal thickness, defined as the vertical distance between the outer surface of the retinal pigment epithelium and the hyperreflective line of the choriocapillary interface, was measured from the horizontal line scan under the center of the fovea. In eyes with

myopic CNV, the choroidal thickness was measured with standard OCT without the EDI technique, as identification of the choriocleral interface in eyes with high myopia is easy and reliable. The central retinal thickness was defined as the distance between the inner surface of the neurosensory retina and the inner surface of the retinal pigment epithelium under the center of the fovea. All measurements were done manually by a retina specialist, blinded to the study parameters, using a built-in caliber OCT machine.

All statistical tests were performed using SPSS version 16.0 software (SPSS Inc, Chicago, IL). All values are presented as mean $\pm$ standard deviation. For statistical analysis, best-corrected visual acuity as measured with a Landolt chart was converted to a logarithm of the minimal angle of resolution. Initial measurement values between the three types of diseases were compared using one-way analysis of variance with Bonferroni corrections. Measurements before and after the treatments were analyzed by repeated measures analysis of variance, and multiple comparisons were done using the Bonferroni post-hoc method. A *P* value of <0.05 was considered statistically significant.

Results

In the current study, 60 eyes of 60 patients (36 men and 24 women; age range, 35–90 years; mean age, 71.1 ± 9.6 years) with subfoveal CNV were treated with ranibizumab. Table 1 shows the initial characteristics of the study population. Table 2 shows the change in mean visual acuity, central retinal thickness, and central choroidal thickness before and after treatments with ranibizumab. Of the 60 eyes studied, 20 had AMD, 20 had PCV, and 20 had myopic CNV. The mean follow-up period was

8.9 ± 3.9 months. The mean refractive error was 1.54 (± 1.75) diopters (D) (range: -1.75 – $+3.0$ D) in the AMD group, -0.56 (± 1.28) (range: -2.75 – $+2.5$ D) in the PCV group, and -10.96 (± 3.32) (range: -21.0 – -7 D) in the myopic CNV group. The mean axial length in eyes with myopic CNV was 28.71 ± 1.01 mm. With ranibizumab treatment, central retinal thickness decreased significantly at 1 month after the loading phase ($P < 0.001$) and at final examination ($P < 0.001$). In parallel with the reduction of retinal thickness, visual acuity was significantly improved at 1 month after the loading phase ($P = 0.005$) and at final examination ($P < 0.001$). However, choroidal thickness (167.2 ± 108.3 μm) showed no significant change at 1 month after the loading phase (165.2 ± 107.8 μm , $P = 0.120$) or at final examination (164.8 ± 107.7 μm , $P = 0.115$) (Table 2).

At baseline, the central retinal thickness in eyes with AMD was significantly greater than in eyes with PCV ($P = 0.005$) or myopic CNV ($P = 0.029$). However, central choroidal thickness in eyes with myopic CNV was significantly lower than in eyes with AMD ($P < 0.001$) or PCV ($P < 0.001$). Immediately after the initiation of treatment, OCT examination showed reduced exudative change in eyes with AMD, PCV, and myopic CNV. Retinal thickness was significantly reduced with the reduction of serous retinal detachment and macular edema (Figures 1–3). Figure 4 shows the change in central retinal thickness in each group. This reduction in retinal thickness was similar in each type of disease. However, OCT examination showed no change in the choroidal structure with the treatments (Figures 1–3). There was no significant change in central choroidal thickness with treatment in any of the disease types (Figure 4).

Table 1 Characteristics of cohort eyes

	Total	Age-related macular degeneration	Polypoidal choroidal vasculopathy	Myopic choroidal neovascularization	<i>P</i> value ^b
Number of eyes	60	20	20	20	
Age (years)	71.1 ± 9.6	76.2 ± 6.2	71.7 ± 9.2	65.6 ± 10.3	0.001
Sex (male/female)	36/24	15/5	16/4	5/15	0.001
Refractive error (diopters) (range)	-2.74 ± 5.97 (-21.0 – $+3.0$)	1.54 ± 1.75 (-1.75 – $+3.0$)	-0.56 ± 1.28 (-2.75 – $+2.5$)	-10.96 ± 3.32 (-21.0 – -7.0)	<0.001
Number of injections (range)	3.2 ± 1.4 (1–7)	3.5 ± 0.9 (3–6)	3.9 ± 1.4 (3–7)	2.2 ± 1.3 (1–5)	<0.001
Follow-up (months)	8.9 ± 3.9	8.4 ± 3.7	8.4 ± 3.3	9.8 ± 4.6	0.422
Visual acuity (logMAR) ^a	0.45 ± 0.37	0.61 ± 0.41	0.24 ± 0.23	0.49 ± 0.37	0.004
Central retinal thickness (μm)	436.0 ± 198.5	551.0 ± 232.5	361.2 ± 161.4	395.9 ± 143.9	0.004
Central choroidal thickness (μm)	167.2 ± 108.3	217.4 ± 98.6	230.8 ± 68.8	53.6 ± 38.2	<0.001

Notes: ^aLogMAR, logarithm of the minimum angle of resolution; ^bvalues were compared using one-way analysis of variance with Bonferroni corrections.

Table 2 Changes in visual acuity, retinal thickness, and choroidal thickness during ranibizumab treatment

	Before treatment	After the loading phase ^{b,c}	At final examination ^c
Visual acuity (logMAR) ^a			
Total	0.45 ± 0.37	0.33 ± 0.38 (0.005)	0.28 ± 0.34 (<0.001)
Age-related macular degeneration	0.61 ± 0.41	0.58 ± 0.43 (0.663)	0.45 ± 0.33 (0.030)
Polypoidal choroidal vasculopathy	0.24 ± 0.23	0.19 ± 0.29 (0.885)	0.12 ± 0.25 (0.046)
Myopic choroidal neovascularization	0.49 ± 0.37	0.23 ± 0.28 (<0.001)	0.24 ± 0.31 (<0.001)
Central retinal thickness (μm)			
Total	436.0 ± 198.5	262.9 ± 113.8 (<0.001)	256.7 ± 106.0 (<0.001)
Age-related macular degeneration	551.0 ± 232.5	298.4 ± 156.6 (<0.001)	293.2 ± 147.8 (<0.001)
Polypoidal choroidal vasculopathy	361.2 ± 161.4	244.7 ± 92.2 (0.008)	236.2 ± 76.1 (0.004)
Myopic choroidal neovascularization	395.9 ± 143.9	245.7 ± 72.1 (<0.001)	240.8 ± 71.7 (<0.001)
Central choroidal thickness (μm)			
Total	167.2 ± 108.3	165.2 ± 107.8 (0.120)	164.8 ± 107.7 (0.115)
Age-related macular degeneration	217.4 ± 98.6	214.2 ± 99.2 (0.305)	215.1 ± 98.7 (0.586)
Polypoidal choroidal vasculopathy	230.8 ± 68.8	228.7 ± 68.0 (0.876)	227.4 ± 67.9 (0.138)
Myopic choroidal neovascularization	53.6 ± 38.2	52.7 ± 38.5 (1.00)	51.9 ± 38.2 (0.348)

Notes: ^aLogMAR, logarithm of the minimum angle of resolution; ^bat the loading phase, all eyes with PCV and AMD received three successive intravitreal injections of ranibizumab at monthly intervals (at baseline and at 1 and 2 months); eyes with myopic CNV were treated by a single injection of ranibizumab; ^cdata were analyzed by repeated measures analysis of variance and multiple comparisons were done using the Bonferroni post-hoc method.

Discussion

An increasing number of investigators have studied the choroid in association with the pathophysiology of various chorioretinal diseases.^{17–24,29,34,35} With the use of EDI-OCT, Chung et al²³ and Koizumi et al²⁴ separately reported foveal choroidal thinning in eyes with exudative AMD. In addition, with the use of 3-dimensional OCT at 1060 nm, Wood et al³⁶ reported choroidal thinning of the macular area in eyes with early AMD. The thinning of the macular choroid may be partially involved in the development of CNV associated with AMD. In eyes with high myopia, previous reports have shown that the choroid is extremely thin;^{29,30} the central choroidal thickness in our patients with high myopia was 53.6 ± 38.1 μm, which was much thinner than in patients with AMD and PCV. In addition, Ikuno et al³¹ reported that eyes with myopic CNV have a thinner choroid than their contralateral eye without CNV; the authors speculated that a thinner choroid is a risk factor for CNV in eyes with high myopia.^{23,24,31}

Treatment with ranibizumab is expected to induce long-term inhibition of all isoforms of VEGF-A in the eye.¹ It may well be that physicians are concerned about the adverse effect of ranibizumab on the choroid, especially when they treat eyes with AMD or high myopia. In the current study, choroidal thickness (167.2 ± 108.3 μm) showed no significant change after the loading phase (165.2 ± 107.8 μm) or at final examination (164.8 ± 107.7 μm). In a PubMed search, we could find no reference to changes in choroidal thickness after anti-VEGF treatment. Based on a retrospective analysis

of 47 eyes with exudative AMD treated with ranibizumab, Sadda et al³⁷ have recently noted that choroidal thickness appears to decrease slightly over time in eyes with exudative AMD, but that ranibizumab does not appear to accelerate this decline. Previously, Margolis and Spaide¹⁸ reported that subfoveal choroidal thickness decreased by 1.56 μm per year in normal subjects. Our patients showed a mean decrease of 2.4 μm (1.4%) in central choroidal thickness during a mean follow-up time of 8.9 months. This decrease in choroidal thickness may not be so different from the change seen in normal subjects.

More recently, Koizumi et al³⁸ reported that in eyes with exudative AMD treated with ranibizumab, mean subfoveal choroidal thickness decreased from 228 μm at baseline to 213 μm at 3 months ($P=0.001$), 215 μm at 6 months ($P=0.009$), and 213 μm at 12 months ($P=0.015$). Although the authors found a statistically significant decrease in the foveal choroidal thickness, the mean reduction was only 15 μm. Recently, Ikuno et al³⁹ reported that the intervisit intraclass correlation coefficient for foveal choroidal thickness was 0.893 (95% CI, 0.864–0.916). However, because measurement of choroidal thickness has high variance due to focal irregularities or indistinctness of the choriocleral border,^{23,24,40,41} it might be difficult to detect minimal changes in thickness practically, even with the EDI-OCT technique. Based on the current findings and other recent reports, the effect of ranibizumab on choroidal thickness is estimated to be small, if any.

In contrast to AMD^{23,24} and high myopia,^{29,30} previous reports showed increased choroidal thickness in PCV.^{23,24,26}

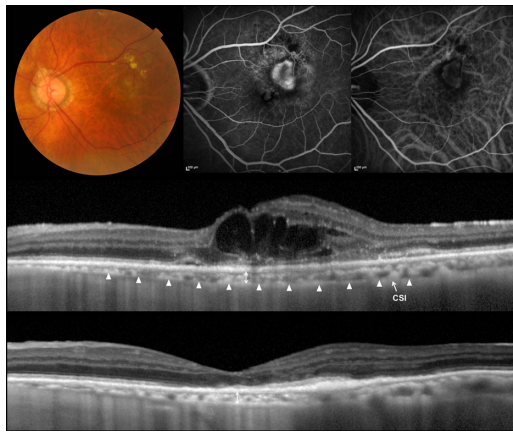


Figure 1 Choroidal neovascularization (CNV) associated with age-related macular degeneration treated with ranibizumab.

Notes: (Top left) Left eye of an 81-year-old man with CNV associated with age-related macular degeneration. Initial visual acuity in the left eye was 0.2 in a Landolt chart (0.70 in logarithm of the minimal angle of resolution) and refractive error was -1.75 Diopter. (Top middle) Fluorescein angiography shows a minimally classic CNV. (Top right) Indocyanine green angiography shows no polypoidal lesions. (Middle) A horizontal section through the fovea obtained by optical coherence tomography with the enhanced-depth imaging technique before the treatment. Multiple cystoid spaces are seen. Central retinal thickness was $529\text{ }\mu\text{m}$, and central choroidal thickness was $105\text{ }\mu\text{m}$. (Bottom) A horizontal section through the fovea after three monthly injections of ranibizumab at the loading phase. Intraretinal fluid was absorbed completely (central retinal thickness, $157\text{ }\mu\text{m}$), and visual acuity improved to 0.3 in a Landolt chart (0.52 in logarithm of the minimal angle of resolution). The choroidal structure shows no changes (central choroidal thickness, $101\text{ }\mu\text{m}$). At 9 months, the central choroidal thickness was still $103\text{ }\mu\text{m}$.

Abbreviation: CSI, choriocapillary interface.

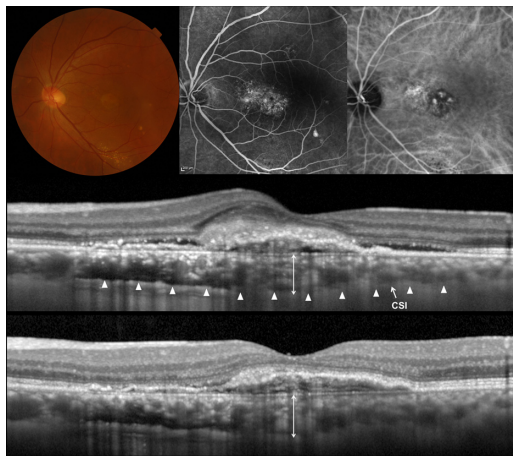


Figure 2 Polypoidal choroidal vasculopathy treated with ranibizumab.

Notes: (Top left) Left eye of a 58-year-old man with polypoidal choroidal vasculopathy. Initial visual acuity in the left eye was 0.15 in a Landolt chart (0.82 in logarithm of the minimal angle of resolution). (Top middle) Fluorescein angiography shows occult with no classic choroidal neovascularization. (Top right) Indocyanine green angiography shows a branching vascular network terminating in polypoidal lesions. (Middle) A horizontal section through the fovea obtained by optical coherence tomography using the enhanced-depth imaging technique before the treatment shows fibrin exudates with surrounding serous retinal detachment. Central retinal thickness was $295\text{ }\mu\text{m}$ and central choroidal thickness was $270\text{ }\mu\text{m}$. (Bottom) A horizontal section through the fovea after three monthly injections and an additional injection of ranibizumab. Exudative change was almost absorbed (central retinal thickness, $235\text{ }\mu\text{m}$), and visual acuity improved to 0.6 in a Landolt chart (0.22 in logarithm of the minimal angle of resolution). The choroidal structure showed no changes, and the central choroidal thickness was $259\text{ }\mu\text{m}$.

Abbreviation: CSI, choriocapillary interface.

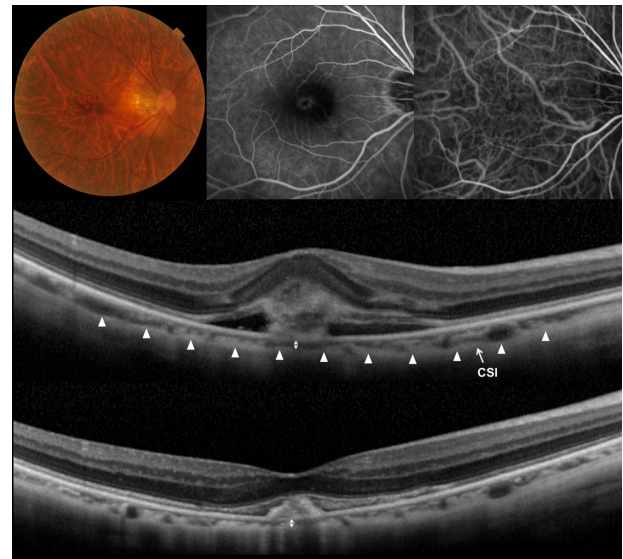


Figure 3 Myopic choroidal neovascularization (CNV) treated with ranibizumab.

Notes: (Top left) Right eye of a 67-year-old woman with CNV secondary high myopia. Spherical equivalent was -8.75 diopters, and axial length was 27.64 mm . Initial visual acuity in the left eye was 0.3 in a Landolt chart (0.52 in logarithm of the minimal angle of resolution). (Top middle and right) Fluorescein (left) and indocyanine green (right) angiography shows a small CNV. (Middle) A horizontal section through the fovea before treatment obtained by optical coherence tomography without the enhanced-depth imaging technique. CNV accompanied exudative change with surrounding serous retinal detachment. Central retinal thickness was $479\text{ }\mu\text{m}$ and central choroidal thickness was $43\text{ }\mu\text{m}$. (Bottom) A horizontal section through the fovea after a single injection of ranibizumab. Intraretinal fluid was absorbed completely (central retinal thickness, $250\text{ }\mu\text{m}$), and visual acuity improved to 0.7 in a Landolt chart (0.15 in logarithm of the minimal angle of resolution). The choroidal structure shows no changes (central choroidal thickness, $40\text{ }\mu\text{m}$).

Abbreviation: CSI, choriocapillary interface.

Recently, Maruko et al²⁶ reported that subfoveal choroidal thickness is decreased after photodynamic therapy in eyes with PCV. In addition, the authors suggested that the choroidal thickening seen in PCV eyes is associated with increased choroidal vascular hyperpermeability.²⁶ After photodynamic therapy, choroidal thickness in eyes with PCV was reduced not only in the macular area, where the photodynamic therapy was performed, but also beyond the vascular arcade. Photodynamic therapy could possibly reduce choroidal thickness by decreasing choroidal vascular hyperpermeability. Our patients with PCV showed no significant reduction in choroidal thickness after ranibizumab treatments. Although VEGF works to increase the vascular permeability in the eye,⁴² the effect of the ranibizumab on choroidal vascular permeability may be limited.

Papadopoulou et al⁴³ recently reported a reduction in the vessel caliber of the retinal arterioles in eyes with AMD after treatment with ranibizumab. In addition, other investigators have reported the vasoconstriction⁴⁴ or even occlusion of retinal arterioles^{45,46} induced by intravitreal injections of bevacizumab, another anti-VEGF agent. Because VEGF

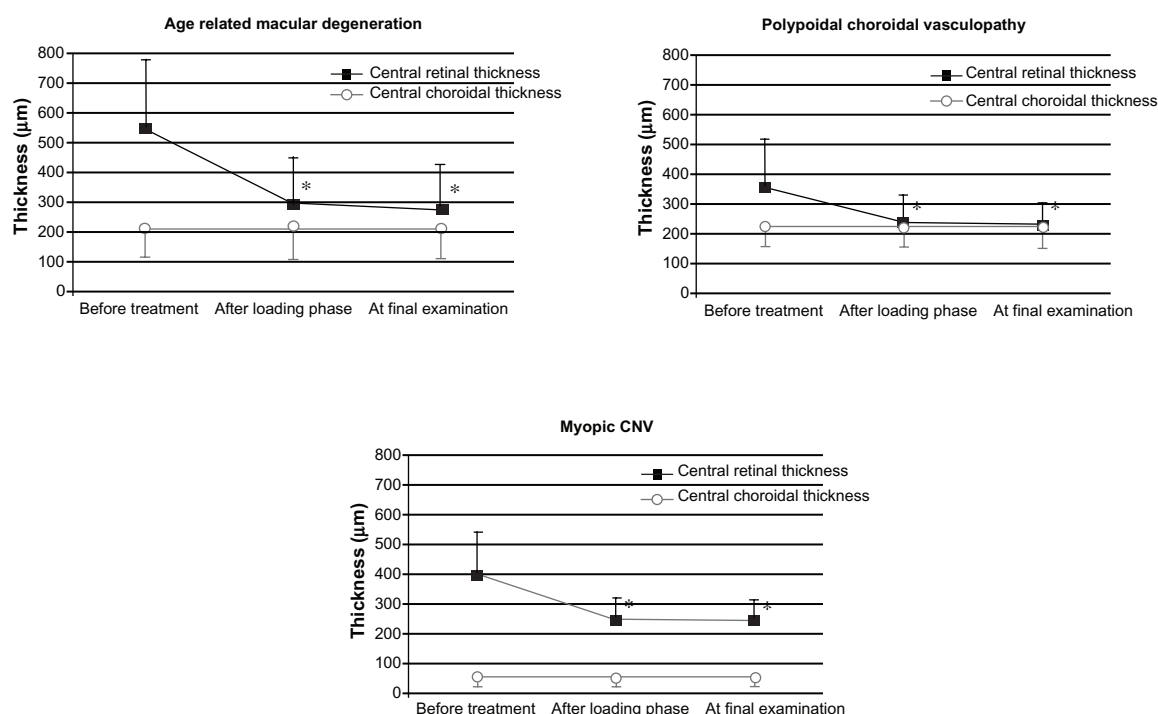


Figure 4 Changes in mean central retinal thickness and mean central choroidal thickness during ranibizumab treatment for choroidal neovascularization (CNV) in eyes with age-related macular degeneration (top left), polypoidal choroidal vasculopathy (top right), and myopic CNV (second row).

Notes: The black squares indicate central retinal thickness; the open circles indicate central choroidal thickness. * $P < 0.01$, compared with values before treatment. At the loading phase, all eyes with PCV and AMD received three successive intravitreal injections of ranibizumab at monthly intervals (at baseline and at 1 and 2 months); eyes with myopic CNV were treated by a single injection of ranibizumab.

is reported to induce vascular dilation and increase retinal blood flow,¹² pan-inhibition of VEGF by ranibizumab may cause vasoconstriction of retinal arterioles. However, we found no prominent effects of ranibizumab on choroidal thickness; it is unclear why no effects were seen. One possibility is that the concentration of ranibizumab in the choroid is low due to the blockage of the retinal pigment epithelium. Another possibility is that VEGF has a limited role in the control of blood flow and vascular permeability in the choroid.

Major limitations of the current study are its small sample size, lack of controls, and short follow-up period. Another limitation is that measurement of choroidal thickness was performed manually and only at the fovea. The measurement of choroidal thickness tends to be affected by focal irregularity or indistinctness of the choriocleral border.^{23,24,40} The raster scan protocol, which uses many measurement points from an OCT at a longer wavelength, would minimize measurement errors. Software to determine the borders of the choroid automatically is essential to standardize this technique further.^{41,47,48} Despite these shortcomings, our findings suggest that the effect of ranibizumab on choroidal thickness can be estimated to be minimal, if any. However,

we studied only the change in the thickness of the choroid and did not evaluate changes in its function. It is possible that choroidal blood flow changes with ranibizumab treatment. Therefore, it may be necessary to evaluate choroid function during ranibizumab treatment as well.

Acknowledgements

This study was supported in part by the Japan Society for the Promotion of Science (JSPS), Tokyo, Japan (Grant-in-Aid for Scientific Research, no 21592256), and by the Japan National Society for the Prevention of Blindness, Tokyo, Japan.

Contributions of the authors were as follows: Conception and design of the study (AAE, AT, NY); analysis and interpretation (AAE, AT, KO, SO, KY, AO, NY); writing of the article (AAE, AT); critical revision of the article (KO, SO, KY, AO, NY); final approval of the article (AAE, AT, KO, SO, KY, AO, NY); and data collection (AAE, AT, KO, SO, KY, AO).

The Institutional Review Board and Ethics Committee of Kyoto University approved this study, which adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from each subject before examination.

Disclosure

The authors report no conflicts of interest in this work.

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